The treatment of lymphoma presents a challenge in clinical oncology. Despite greater tumor cytoreduction achieved with increased doses and new combinations of chemotherapeutic agents, a large number of patients eventually relapse. It is believed that one factor that limits the effectiveness of chemotherapy is the induction of intrinsic resistance of tumor cells resistant to chemotherapy. Resistance to a wide variety of natural-product antineoplastic drugs, such as anthracyclines, Vinca alkaloids, etoposide, and actinomycin D is often because of the phenomenon known as multidrug resistance (MDR). MDR results from reduced intracellular drug accumulation in association with overexpression of the mdr gene encoding a membrane glycoprotein, termed P-glycoprotein, which mediates transport of drugs out of the cells.

The following agents have different mechanisms of action and are not subject to toxic plant lectin ricin, called blocked ricin, which has its role are immunotoxins, conjugates between monoclonal antibodies (MoAb) and potent toxins. Immunotoxins for the treatment of leukemias and lymphomas have already proceeded through clinical trials.

Anti-B4-blocked ricin (anti-B4-bR) is an immunotoxin comprised of an anti-CD19 MoAb and a derivative of the toxic plant lectin ricin, called blocked ricin, which has its galactose-binding sites blocked by covalently linked affinity ligands to minimize nonspecific toxicity. The CD19 antigen is expressed on normal and neoplastic B-cells, is B-lineage restricted, and is detected early in B-cell ontogeny. Anti-B4-bR has shown cytotoxic potency, selectivity, and specificity in vitro and in vivo. It has shown antitumor effects in SCID mouse models of human CD19+ B-cell neoplasms. Phase I dose escalation trials have shown safety and efficacy at therapeutic efficacy for patients with relapsed and refractory B-cell neoplasms.

To develop a rationale for combining anti-B4-bR with chemotherapeutic drug regimens we studied the interaction of the immunotoxin with doxorubicin and etoposide on the drug-sensitive Burkitt's lymphoma cell line Namalwa and the multidrug-resistant cell line Namalwa/mdr-1. Anti-B4-bR showed synergistic killing with both of these drugs on the sensitive and drug-resistant Namalwa cells in vitro. Treatment of the Namalwa/mdr-1 xenografts in vivo with anti-B4-bR could completely reverse the drug-resistant phenotype and render the tumors as sensitive to either doxorubicin or etoposide as the Namalwa tumors. In this study these observations were extended to show that anti-B4-bR synergizes with the alkylating agents 4-hydroperoxy-cyclophosphamide (4-HC) and cisplatin in vitro on both Namalwa and Namalwa/mdr-1 cells. Furthermore, it was found that anti-B4-bR could cure the Namalwa/mdr-1 tumor-bearing animals when used in combination with multidrug regimens. The two drug regimens, CHOED and CCE, are experimental multidrug regimens used in combination with current multidrug regimens may constitute a highly efficacious modality for the treatment of drug-resistant B-cell malignancies.
To avoid excessive toxicity, the total doses of each agent were maintained as described previously. Both the drug-sensitive (1.7 \times 10^4 cells/well) and drug-resistant cells (1.2 \times 10^4 cells/well) were incubated in the presence of various concentrations of anti-B4-bR, vincristine, doxorubicin, etoposide, cisplatin, or 4-HC for 72 hours. Surviving cells were then assayed by exposure to [\textsuperscript{3}H]-thymidine (1 \muCi/well) for 18 hours followed by counting the radioactivity incorporated into precipitated polymers. Cultures not exposed to drugs served as controls. Data were calculated as average counts per minute (cpm) from three experiments, each performed in 96-well plates using triplicate wells for each data point. Dose response curves were generated based on expressing cpm from treated cells as a fraction of cpm from untreated controls. IC_{50} values (defined as the concentration of drug that reduced [\textsuperscript{3}H]dT by 63%) were determined graphically from relative survival curves. The in vitro data were analyzed using an adaptation of isobologram methodology used in the case where one agent is held constant as described previously. Envelopes of additivity are derived from mode I additivity which accounts for potency differences between the two agents and mode II additivity, which is derived from the linear isoeffect relationship. Combinations producing an affect within the envelope boundaries of mode I and mode II are considered to be additive; those displaced to the left are greater than additive (ie, supra-additive), whereas those displaced to the right are less than additive (ie, subadditive).

Animals and tumor models. Female CB-17 SCID mice were obtained from Massachusetts General Hospital (Boston, MA) and were quarantined for at least 1 week. Animals were housed on a 12-hour light-dark cycle with food and water available ad libitum. Cages, bedding, food, and water were sterilized. Water was acidified with 1N hydrochloric acid to pH 2.4 to suppress growth of Pseudomonas. When the mice were 6 to 7 weeks of age, a suspension (0.2 mL) of Namalwa/mdr-1 or Namalwa cells (4 \times 10^6 cells) was injected into a tail vein of the animals. This procedure resulted in a 100% tumor take with untreated or PBS-treated animals dying over a 5 day range with a median survival time (MST) of about 25 days after tumor inoculation. Necropsy of tumor-bearing animals showed that the tumors have tropism for the ovaries, leptomeninges of the brain, bone marrow (BM) of the vertebra, dura mater of the spinal cord, and the muscles surrounding the spinal column.

Treatment techniques. Anti-B4-bR was diluted appropriately with phosphate-buffered saline (PBS), pH 7.4, containing 0.1% human serum albumin (HSA), and injected into a tail vein of tumor bearing animals. The chemotherapeutic drugs vincristine, doxorubicin, etoposide, cyclophosphamide or cisplatin were administered intravenously (i.v.). Preliminary studies indicated that sequential treatments with anti-B4-bR and chemotherapeutic drugs were more effective than concurrent administration. Therefore, the combination therapies with anti-B4-bR and the multidrug regimens were conducted by treatment with the immunotoxin for 5 days starting on day 7 after tumor inoculation, followed by the multidrug regimens. To avoid excessive toxicity, the total doses of each agent were administered at about 45% to 65% of their maximum tolerated doses (MTDs) and the administration schedule altered so that the mice did not decrease in body weight more than 20% during the therapy. The MTD was defined as the maximum dose that could be administered to tumor-bearing mice without resulting in drug-related death. Optimal doses for the multidrug regimens were developed which gave maximal therapeutic benefit without deaths in the treated groups of mice. Control animals received no treatment, as we have observed previously that treatment with vehicle solution (PBS containing 0.1% HSA) did not alter the survival of the tumor-bearing mice. A group receiving anti-B4-bR alone, as well as a group receiving the multidrug regimen alone at their respective optimal treatment schedules were included in each experiment as positive controls.

Endpoints and data analysis. Each group contained 8 to 10 tumor-bearing animals. The endpoint for this study was animal survival. Animals were checked daily and animals that showed deteriorating and moribund condition were euthanized with CO2. The survival of all mice was followed and an MST calculated for each group. The increase in life-span (ILS) was calculated by dividing the MST of a treatment group by the MST of the control group and is expressed as the percent increase over the life-span of the control animals. Statistical analysis was performed by the log-rank test and the Wilcoxon test at the 5% significance level.

RESULTS

Combinations of anti-B4-bR and cisplatin or 4-HC on Namalwa and Namalwa/mdr-1 cells. The sensitivity of Namalwa and Namalwa/mdr-1 to anti-B4-bR and chemotherapeutic drugs in vitro has been previously reported. Namalwa cells were slightly more resistant than Namalwa mdr-1 cells to cisplatin with IC_{50} values of 250 nmol/L and 170 nmol/L, respectively, whereas both cell lines had an IC_{50} of 1 \muM of 4-HC. Namalwa cells were also slightly more resistant than Namalwa/mdr-1 cells to anti-B4-bR with IC_{50} values of 8 pmol/L and 4 pmol/L, respectively. Namalwa/mdr-1 cells were approximately sixfold more resistant to doxorubicin and etoposide, and 40-fold more resistant to vincristine, showing a functional MDR phenotype.

To study the cytotoxic effects of anti-B4-bR combined with cisplatin, Namalwa and Namalwa/mdr-1 cells were incubated with anti-B4-bR at concentrations of 10 pmol/L or 20 pmol/L and treated simultaneously with cisplatin at concentrations ranging from 0.1 to 1.0 \muM in the case of Namalwa cells and 0.1 to 0.5 \muM in the case of Namalwa/mdr-1 cells. The cytotoxicity was measured by [\textsuperscript{3}H]dT incorporation assays and the killing curves were subjected to isobologram analysis. The resultant isobolograms with the calculated envelopes of additivity are shown in Fig 1A (Namalwa) and Fig 1B (Namalwa/mdr-1). They indicate additive killing with the lower concentration of anti-B4-bR (10 pmol/L) for each cell line. At the higher concentration of anti-B4-bR (20 pmol/L), the killing curves lie significantly outside the envelope of additivity in the area of supra-additivity.

The cytotoxic effects of combinations of anti-B4-bR and 4-HC were assessed in a similar way. Namalwa and Namalwa/mdr-1 cells were incubated with anti-B4-bR at concentrations of 10 pmol/L and 20 pmol/L. Each immunotoxin level was combined with 4-HC concentrations ranging from 0.5 to 3 \muM or 0.5 to 2 \muM for Namalwa and Namalwa/mdr-1 cells, respectively. Isobolograms obtained are
shown in Fig 2A (Namalwa) and Fig 2B (Namalwa/mdr-1). The killing curves for anti-B4-bR at both 10 pmol/L and 20 pmol/L concentrations show additive killing towards Namalwa cells. Namalwa/mdr-1 cells show additive killing at 10 pmol/L and supra-additive killing at 20 pmol/L Anti-B4-bR.

Anti-B4-bR and chemotherapy in treatment of the MDR tumor Namalwa/mdr-1. The finding of additive or synergistic killing with anti-B4-bR and cisplatin or 4-HC on Namalwa/mdr-1 cells, coupled with our previously reported synergy between Anti-B4-bR and doxorubicin, etoposide, vincristine on these cell lines in vitro and in vivo encouraged us to explore the possibility of combining anti-B4-bR with multidrug regimens consisting of both MDR-related drugs and non-MDR-related drugs. In support of these studies, well-tolerated doses and efficacy data for all of the chemotherapeutic drugs as single agents had to be established for the Namalwa tumor model in SCID mice.

The therapeutic efficacy of anti-B4-bR and the chemotherapeutic drugs cyclophosphamide, cisplatin, vincristine, doxorubicin and etoposide, as single agents in the treatment of Namalwa or Namalwa/mdr-1 xenograft tumors are summarized in Table 1 and Table 2, respectively. All agents at their respective MTD were effective in prolonging survival of the
Namalwa tumor burdened-mice. Administration of anti-B4-bR alone beginning on day 7 after tumor inoculation resulted in an MST of 38 days or an ILS of 65% over the life span of untreated control animals (MST = 23 days). Treatment with cyclophosphamide alone produced an MST of 44 days (ILS = 91%). Vincristine, doxorubicin and etoposide were somewhat less effective: therapy with each of the three drugs as single agents resulted in MST values of 28 days (ILS = 22%), 29 days (ILS = 26%), and 30 days (ILS = 32%), respectively.

Namalwa/mdr-1 tumor-bearing mice treated with five daily bolus iv injections of anti-B4-bR resulted in an MST value of 43 days which, when compared to the control MST of 25 days, corresponds to an ILS of 72% (Table 2). Vincristine, doxorubicin or etoposide as single agents did not show significant effects on survival of the Namalwa/mdr-1 tumor-burdened mice. However, treatment with both cyclophosphamide and cisplatin, drugs that are not subject to MDR, were effective in prolonging survival of treated animals with MSTs of 48 days (ILS = 92%) and 32 days (ILS = 28%), respectively. Noteworthy in this study as well as in our previous study is that the Namalwa/mdr-1 tumor is more responsive to anti-B4-bR than the drug-sensitive Namalwa tumor.

Combination therapies of the MDR tumor Namalwa/mdr-1 with anti-B4-bR and multidrug regimens. We next explored the possibility of combining anti-B4-bR with multidrug regimens, which would be used in a clinical setting and consist of the drugs used above. Based on the data with reversal of drug resistance in the Namalwa/mdr-1 cells, and because synergism with the alkylating drug 4-HC and cisplatin was observed on both Namalwa and Namalwa/mdr-1 cells, two multidrug regimens, CHOE and CCE were investigated in SCID/Namalwa/mdr-1 model.

In the anti-B4-bR-CHOE study, Namalwa/mdr-1 tumor-bearing mice were treated either with anti-B4-bR alone, with CHOΕ alone, or with anti-B4-bR plus CHOE (Fig 3). Although treatment with an optimal dose of the multidrug regimen alone or treatment with anti-B4-bR alone (50 µg/kg/d × 5, 67% of the MTD) could significantly increase survival of the tumor-burdened mice (MSTs = 41 and 45 days, respectively, compared to the MST of 26 days for the control group; ILS of 58% and 73%, respectively; P < .001), there were no cures. However, treatment with anti-B4-bR followed by the same doses of CHOE resulted in an MST of 71 days for treated mice (ILS = 173%) and produced long-term cures in 2 of 10 mice (animals survived over 180 days at which time the experiment was terminated, Fig 3). When gross and histopathologic examinations were performed, neither of these mice had any sign of tumor.

An even better result was observed in mice when treatment with anti-B4-bR was combined with the CCE regime (Fig 4). In this study Namalwa/mdr-1 tumor-bearing mice were treated either with anti-B4-bR alone, with the CCE regimen alone, or with anti-B4-bR plus CCE. Treatment with the optimal dose of CCE alone was effective in increasing the MST of the animals from 24 days (control group) to 55 days (ILS = 129%; P < .0005). Anti-B4-bR alone increased the MST to 40 days (ILS = 67%, P < .001). The combination therapy of anti-B4-bR plus CCE was extremely effective.

<table>
<thead>
<tr>
<th>Table 1. Anti-B4-bR and Chemotherapy in Treatment of the Drug-Sensitive Namalwa Tumor in a SCID Mouse Model</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Treatment</strong></td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Untreated control</td>
</tr>
<tr>
<td>Anti-B4-bR (75 µg/kg x 5, d7-11)</td>
</tr>
<tr>
<td>Cyclophosphamide (100 mg/kg x 3, d7, 9, 11)</td>
</tr>
<tr>
<td>Vincristine (400 µg/kg x 3, d7, 9, 11)</td>
</tr>
<tr>
<td>Doxorubicin (3 mg/kg x 3, d7, 11, 15)</td>
</tr>
<tr>
<td>Etoposide (15 mg/kg x 3, d7, 9, 11)</td>
</tr>
</tbody>
</table>

* Treatment started on day 7 after tumor inoculation. Each group included 8 to 10 tumor-bearing mice.
† The survival range of animals in each group is given in parentheses.
‡ Comparison with control group by Wilcoxon analysis at the 5% significance level.

<table>
<thead>
<tr>
<th>Table 2. Anti-B4-bR and Chemotherapy in Treatment of the Drug-Resistant Namalwa/mdr-1 Tumor in a SCID Mouse Model</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Treatment</strong></td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Untreated control</td>
</tr>
<tr>
<td>Anti-B4-bR (75 µg/kg x 5, d7-11)</td>
</tr>
<tr>
<td>Cyclophosphamide (100 mg/kg x 3, d7, 9, 11)</td>
</tr>
<tr>
<td>Cisplatin (5 mg/kg x 3, d7, 11, 15)</td>
</tr>
<tr>
<td>Vincristine (400 µg/kg x 3, d7, 9, 11)</td>
</tr>
<tr>
<td>Doxorubicin (3 mg/kg x 3, d7, 11, 15)</td>
</tr>
<tr>
<td>Etoposide (15 mg/kg x 3, d7, 9, 11)</td>
</tr>
</tbody>
</table>

Abbreviation: NS; not significant.
* Treatment started at day 7 after tumor inoculation. Each group included 8 to 10 tumor-bearing mice.
† The survival range of animals in each group is given in parentheses.
‡ Comparison with control group by Wilcoxon analysis at the 5% significance level.
and produced long-term cures in 50% of the tumor-burdened mice. Again these mice showed no signs of tumor, confirmed by histopathologic examination on day 180.

**DISCUSSION**

While the testing of immunotoxins alone in the treatment of leukemias and lymphomas is proceeding rapidly in clinical trials, the evaluation of immunotoxins in combination with chemotherapy may lead to other promising therapeutic applications. Several investigations including our in vitro and in vivo studies have shown synergistic or additive effects of immunotoxins and chemotherapeutic drugs in treatment of certain types of tumors. These studies were focused on drug-sensitive tumors.

In previous studies, we have shown that anti-B4-bR could reverse multidrug resistance of Namalwa/mdr-1 cells to doxorubicin, etoposide, and vincristine in vitro and in vivo. In the current study anti-B4-bR produced greater than additive cytotoxicity with the antitumor alkylating drugs 4-HC and cisplatin against both drug-sensitive and drug-resistant tumor cells. The observation that synergistic killing was obtained with these agents on both Namalwa and Namalwa/mdr-1 cells showed that this synergism is independent of the MDR phenotype. This coupled with our previous observation of synergistic cytotoxicity between anti-B4-bR and doxorubicin, etoposide, or vincristine against MDR expressing cells may suggest that anti-B4-bR can synergize with drugs by downregulating several drug-resistance related proteins (such as P-glycoprotein, glutathione synthetase, and glutathione S-transferase) through its action of inhibiting protein synthesis. This indicates that the immunotoxin could be used to good effect when added to chemotherapeutic regimens consisting of both MDR-related and non-MDR-related drugs.

When it was established that anti-B4-bR could interact positively with these different drugs, two multidrug regimens, which are used for the treatment of refractory non-Hodgkin’s lymphomas, were modeled in animals. It is likely that a combination study with the immunotoxin and multidrug regimens would be a more realistic model for the clinical situation and would provide important information for the design of a clinical trial. The combination therapies of anti-B4-bR plus the single agents vincristine, doxorubicin, or etoposide or multidrug regimens (without need to reduce the drug doses) could be tolerated by the tumor-bearing mice. However, in the combination therapies, the intervals of administration of the chemotherapeutic drugs had to be prolonged, because the initial studies showed that using the same treatment schedules as single agent treatment caused unacceptable systemic toxicity as shown by a body weight loss of >20% with several animals dying of toxicity.

An interesting feature of these studies was that although the drug combinations alone without the immunotoxin were effective therapy against the Namalwa/mdr-1 tumor by significantly extending the lifespan of the mice, they could still be improved upon by the immunotoxin anti-B4-bR to bring about cures. This shows how existing drug combinations could be further improved by adding a therapeutic agent such as anti-B4-bR, which does not have overlapping toxicity and is not subject to the same drug-resistant mechanisms as the agents in the existing combinations.

The importance of overcoming the MDR phenotype for a successful cancer treatment has been firmly established.

![Graph showing combination therapy with anti-B4-bR and multidrug regimen CHOE of Namalwa/mdr-1 tumors in SCID mice. The survival of mice as a function of time after Namalwa/mdr-1 tumor inoculation (day 0) is shown. Animals were treated either (A) with anti-B4-bR alone (50 µg/kg x 5, d7-11); (B) with the CHO regimen alone (cyclophosphamide 75 mg/kg x 2, d7, 10; doxorubicin 3 mg/kg x 2, d7, 10; vincristine 300 µg/kg x 2, d6, 19; etoposide 10 mg/kg x 2, d8, 19); or (C) combination therapy with anti-B4-bR (50 µg/kg x 5, d7-11) followed by CHO (cyclophosphamide 75 mg/kg x 2, d14, 27; doxorubicin 3 mg/kg x 2, d14, 27; vincristine 300 µg/kg x 2, d15, 28; etoposide 10 mg/kg x 2, d15, 28). (C) Control animals received no treatment. Each group contained 10 tumor-burdened mice.](image)
Several agents, including verapamil, quinidine and amiodarone, rifampicin, chloroquine, progesterone, cyclosporine, tamoxifen, MoAbs, and immunotoxins directed specifically against P-glycoprotein are able to modulate the activity of P-glycoprotein in vitro. However, their clinical utility may be limited because of their other pharmacological activities and their associated toxicities. For example, whereas cyclosporine may have some utility for leukemia, the calcium-channel blocker verapamil has been shown to enhance cytotoxicity by increasing the intracellular accumulation of drugs, but only at concentrations that would result in excessive cardiac toxicity. The two antiarrhythmic drugs, quinidine and amiodarone, have also entered clinical trials as chemo sensitizers. Both drugs have also produced a number of adverse clinical side effects.

Anti-B4-BR overcomes this hurdle in the sensitization of the MDR B-cell lymphoma. It is a cytotoxic agent with an activity selective for CD19-expressing cells that can be safely used in humans. A clinical trial is currently underway that takes advantage of the ability of anti-B4-BR to reverse the MDR phenotype and to synergize in vitro and in vivo with non-MDR-related drugs (cyclophosphamide and cisplatin).

ACKNOWLEDGMENT

We are grateful to Susan M. Derr, Cynthia A. Ferris, Christopher Corvi, and Tammy Davis for technical assistance. We thank Dr. Roderick T. Bronson (School of Veterinary Medicine, Tufts University, North Grafton, MA) for the histopathologic studies.

REFERENCES

27. Teicher BA, Hermann TS, Holder SA, Eder JP: Chemotherapeutic potentiation through interaction at the level of DNA, in Chou


For personal use only.on August 16, 2017. For personal use only.
Cure of multidrug-resistant human B-cell lymphoma xenografts by combinations of anti-B4-blocked ricin and chemotherapeutic drugs

C Liu, JM Lambert, BA Teicher, WA Blattler and R O'Connor